

## An Endogenous Inhibitor of Focal Adhesion Kinase Blocks Rac1/JNK but Not Ras/ERK-dependent Signaling in Vascular Smooth Muscle Cells\*

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**Humoral factors and extracellular matrix are critical co-regulators of smooth muscle cell (SMC) migration and proliferation. We reported previously that focal adhesion kinase (FAK)-related non-kinase (FRNK) is expressed selectively in SMC and can inhibit platelet-derived growth factor BB homodimer (PDGF-BB)-induced proliferation and migration of SMC by attenuating FAK activity. The goal of the current studies was to identify the mechanism by which FAK/FRNK regulates SMC growth and migration in response to diverse mitogenic signals. Transient overexpression of FRNK in SMC attenuated autophosphorylation of FAK at Tyr-397, reduced Src family-dependent tyrosine phosphorylation of FAK at Tyr-576, Tyr-577, and Tyr-881, and reduced phosphorylation of the FAK/Src substrates Cas and paxillin. However, FRNK expression did not alter the magnitude or dynamics of ERK activation induced by PDGF-BB or angiotensin II. Instead, FRNK expression markedly attenuated PDGF-BB-, angiotensin II-, and integrin-stimulated Rac1 activity and attenuates downstream signaling to JNK. Importantly, constitutively active Rac1 rescued the proliferation defects in FRNK expressing cells. Based on these observations, we hypothesize that FAK activation is required to integrate integrin signals with those from receptor tyrosine kinases and G protein-coupled receptors through downstream activation of Rac1 and that in SMC, FRNK may control proliferation and migration by buffering FAK-dependent Rac1 activation.**

Smooth muscle cell (SMC)<sup>1</sup> proliferation is certainly important during vascular development, but it is clear that increased

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<sup>1</sup> The abbreviations used are: SMC, smooth muscle cell; PDGF, platelet-derived growth factor; PBS, phosphate-buffered saline; Ab, antibody; ECM, extracellular matrix; Ang II, angiotensin II; FAK, focal adhesion kinase; FRNK, FAK-related non-kinase; GFP, green fluorescent protein; CAS, p130 Crk-associated substrate; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; PAK, p21-activated kinase 1; JNK, c-Jun N-terminal kinase; BrdUrd, bromodeoxyuridine; GFRNK, cells expressing GFP-FRNK; IGF-1, insulin-like growth factor-1; ET-1, endothelin-1; DsV, pDsRedC1 vector; DsRacL61, pDsRedC1-tagged RacL61; GST, glutathione S-transferase; pY, phosphorytyrosine.

SMC proliferation and migration are important contributors to the pathogenesis of several important cardiovascular disease states including atherosclerosis, restenosis, and hypertension. A large number of extrinsic cues (growth factors, extracellular matrix, cell-cell interactions, etc.) have been identified that regulate SMC growth and migration (1, 2). However, the precise cellular signaling mechanisms involved are not completely understood, and very little is known about how (or if) these signaling pathways are integrated.

The majority of soluble SMC mitogens can be broadly divided into two groups, activators of receptor tyrosine kinases (*i.e.* platelet-derived growth factor BB homodimer (PDGF-BB), basic fibroblast growth factor, insulin-like growth factor-1 (IGF-1), and activators of G protein-coupled receptors (*i.e.* angiotensin II (Ang II), thrombin, endothelin-1 (ET-1); see Ref. 2). A large body of evidence indicates that members of both groups activate (to varying degrees) the Ras/Raf/MEK/ERK and PI3K/Rac/PAK/JNK kinase cascades, phospholipase C, and protein kinase C signaling pathways, among others (1). Interestingly, it is becoming clear that the mitogenic responses elicited by many of these factors are dependent upon extracellular matrix (ECM)/integrin interactions. For example, when cultured fibroblasts are held in suspension, PDGF-, epidermal growth factor-, and lysophosphatidic acid-stimulated ERK activity is markedly reduced compared with cells plated on fibronectin (3, 4).

Integrin-mediated activation of the non-receptor tyrosine kinase, focal adhesion kinase (FAK), is a critical step in integrin signaling and may be important for growth factor signaling, as well. In support of this idea, several mitogens (including PDGF-BB, Ang II, IGF-1, and ET-1) stimulate FAK tyrosine phosphorylation in an adhesion-dependent manner (5–8). In addition, Renshaw *et al.* (9) have shown that integrin augmentation of growth factor-mediated signaling to ERK2 appears to be dependent upon FAK activation, because the inhibition of mitogen-activated protein kinase activation observed in suspended cells can be rescued by overexpression of activated FAK. Furthermore, integrin-mediated autophosphorylation of FAK on Tyr-397 leads to recruitment and activation of Src and PI3K and subsequent downstream activation of Ras/ERK or Rac/JNK cascades (10, 11).

Interestingly, our laboratory showed recently that FAK appears to be regulated in a unique fashion in SMC. We reported that the noncatalytic C-terminal domain of FAK, termed FAK related non-kinase (FRNK), is selectively expressed in SMC with very high levels found in the large arterioles. FRNK transcription results from the utilization of an alternative start site within the FAK gene, and FRNK expression is independently regulated by a distinct promoter embedded within FAK intronic sequences (12, 13). Interestingly, whereas FAK protein levels remain relatively constant, FRNK protein levels *in vivo*

are dynamically regulated with increased FRNK expression in the neonatal period and from 2 to 3 weeks following balloon injury. This pattern of expression correlates with the attenuation of SMC proliferation that is known to occur under these circumstances, suggesting that FRNK may be an important regulator of SMC growth (2). In support of this idea, we have shown that overexpression of GFP-FRNK in rat aortic SMC completely prevented the PDGF-BB-induced increase in [<sup>3</sup>H]thymidine incorporation and significantly inhibited the mitogenic effects of serum. In addition, GFP-FRNK also significantly inhibited fibronectin-dependent SMC migration toward PDGF-BB (12). Taken together these data suggest that FRNK acts as an endogenous inhibitor of FAK activity and that its expression *in vivo* may act to buffer FAK-dependent proliferative and migratory signals.

In this report we sought to identify the mechanism by which FRNK attenuates growth factor and adhesion signaling in SMC. Our data indicate that in contrast to other cell types, FAK/FRNK signaling does not modify agonist-stimulated ERK activity in SMC. Instead, our data reveal that in SMC, Rac1 activation may be a key convergence point in growth factor- and FAK-dependent integrin-regulated cell proliferation.

#### EXPERIMENTAL PROCEDURES

**Antibodies and Reagents**—The 4G10 phosphotyrosine-specific antibody, an anti-human FAK antibody, and the Rac1 antibody were purchased from Upstate Biotechnology, Inc. The phosphotyrosine-specific anti-FAK antibodies (Tyr-397, Tyr-576, Tyr-577, and Tyr-861) were purchased from BioSource International. Texas Red-conjugated phalloidin was purchased from Molecular Probes. The phosphospecific and immobilized phospho-p44/42 mitogen-activated protein kinase antibody was purchased from Cell Signaling, along with the phospho-stress-activated protein kinase/JNK (Thr-183/Tyr-185) and phospho-ELK-1 antibodies. CAS was detected using a mixture of two polyclonal antibodies generated against the C-terminal domain of CAS (CAS-F and CAS-P; provided by Dr. Amy Bouton, University of Virginia) (14). The monoclonal ERK1/2 antibody (1B3B9) was generously provided by Dr. Michael Weber (University of Virginia). The anti-FLAG (M5) antibody, Ang II, and fibronectin were purchased from Sigma, and PDBF-BB was purchased from Calbiochem.

**Adenovirus Production and Expression Constructs**—The GFP and GFP-tagged FRNK viruses (replication-defective  $\psi$ 5 adenovirus) were plaque-purified by cesium chloride gradients as described previously (12). The cDNA construct encoding an N-terminal DsRed-conjugated L61Rac1 was generated by cloning L61Rac1 into the 5' *Bam*HI and 3' *Eco*RI restriction sites in the pDsRed2-CI vector (Clontech).

**Cell Culture, Infection, Transfection, and Agonist Treatment**—Rat aortic SMC were obtained from 8-week rat thoracic aortas by enzymatic digestion as described previously (15). Cells were used from passages 7–21 and were maintained in Dulbecco's modified Eagle's medium-F-12 (1:1) plus 10% fetal bovine serum and 1% penicillin-streptomycin.

For adenoviral infection, cells were incubated in serum containing medium with either GFP or GFP-FRNK (MOI 10) for 12–15 h prior to treatment. In some experiments, cells were serum-starved for 4 h followed by treatments with PDGF-BB or Ang II for the times indicated. For the adhesion suspension experiments, cells were trypsinized, neutralized in soybean trypsin inhibitor (1 mg/ml in PBS), collected by centrifugation, and resuspended in serum-free Dulbecco's modified Eagle's medium-F-12. Cells were held in suspension or plated on fibronectin-coated (40  $\mu$ g/ml) dishes for the times indicated. In some experiments, SMC were transfected using Gene Jammer transfection reagent (Stratagene) following the manufacturer's instructions.

**Immunocytochemistry**—SMC were plated on Lab Tek II chamber slides (5,000 cells/cm<sup>2</sup>; Nalgene) and infected with virus, as described above. Cells were serum-starved for 4 h (Dulbecco's modified Eagle's medium-F-12, 1% penicillin-streptomycin) and then dosed with 20 ng/ml PDGF-BB for 45 min. Slides were then washed three times with PBS (calcium- and magnesium-free) and fixed in 4% paraformaldehyde in PBS for 20 min. Following an additional three washes in PBS, slides were permeabilized for 3 min with 4% Triton X-100 in PBS. Detergent was removed by washing three times with PBS and then slides were blocked with blocking solution (5% goat serum, 2% bovine serum albumin in PBS) for 30 min. Cells were incubated for 1 h with Texas Red

phalloidin in PBS (1:1000) to visualize filamentous actin. All steps were performed at room temperature.

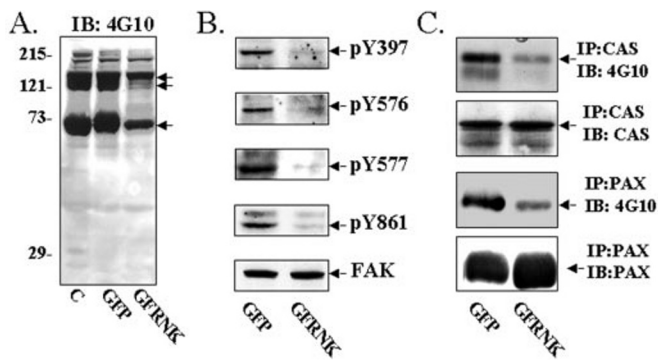
**BrdUrd and WST-1 Proliferation Assays**—To detect BrdUrd incorporation, SMC plated on Lab Tek II chamber slides, as described above, were transfected with 0.7  $\mu$ g of pDsRedC1 vector (DsV) or pDsRedC1-tagged RacL61 (DsRacL61) using Gene Jammer transfection reagent. 48 h following transfection, cells were infected with GFP or GFRNK adenoviruses for 15 h. Cells were serum-starved as described above and then treated with either 10% fetal bovine serum or PDGF-BB (30 ng/ml) overnight. Cells were then incubated with 1/1000 dilution of BrdUrd labeling reagent (Roche) for 1 h at 37 °C. Slides were washed three times for 5 min with PBS and then fixed with ethanol (50 mM glycine, 70% ethanol, pH 2.0) in the dark at –20 °C for 20 min. BrdUrd detection reagent was used following the manufacturer's instructions, followed by a 30-min incubation at 37 °C with a cascade blue-conjugated secondary antibody (1:1000; Molecular Probes). Slides were washed and mounted with coverslips, and BrdUrd-positive nuclei were visualized by indirect fluorescence microscopy. For the WST-1 experiments, SMC were plated in 96-well culture dishes (2,000 cells/well) and treated as described above, with the exception that the WST-1 tetrazolium salt (Roche Applied Science) was added to the culture for 2 h to monitor cell proliferation as per the manufacturer's instructions. After this incubation period the production of formazan dye was quantitated using a spectrophotometer (450 nm).

**Immunoprecipitation and Western Blotting**—SMC were lysed in modified radioimmune precipitation assay buffer (50 mM Hepes, 0.15 M NaCl, 2 mM EDTA, 0.1% Nonidet P-40, 0.05% sodium deoxycholate, pH 7.2) containing 1 mM Na<sub>3</sub>VO<sub>4</sub>, 40 mM NaF, 10 mM Na<sub>2</sub> pyrophosphate, 100  $\mu$ M leupeptin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.02 mg/ml soybean trypsin inhibitor, and 0.05 trypsin inhibitory units/ml aprotinin. CAS and paxillin were immunoprecipitated by incubation of 1 mg of cell extract with 5  $\mu$ g of the appropriate antibody for 2 h at 4 °C, followed by a 1-h incubation with protein A-Sepharose-conjugated beads (Amersham Biosciences). For paxillin immunoprecipitations, the beads were pre-coupled rabbit anti-mouse Ab (10  $\mu$ g/ml; Jackson Laboratories). The immune complexes were collected by centrifugation and washed three times with radioimmune precipitation assay buffer and two times with Tris-buffered saline (0.2 M NaCl, 50 mM Tris-HCl, pH 7.4). Proteins were boiled in sample buffer and resolved on a 10% SDS-PAGE and transferred to nitrocellulose. Western blots were performed using the appropriate antibodies at a 1/1000 dilution (except anti-ERK, 1B3B9, which was used at 1:10,000). Blots were washed in Tris-buffered saline plus 0.05% Triton-X, followed by incubation with either horseradish peroxidase-conjugated protein A-Sepharose (Amersham Biosciences) or horseradish peroxidase-conjugated rabbit anti-mouse antibody (Amersham Biosciences) at a 1/2000 dilution. Blots were visualized after incubation with chemiluminescence reagents (ECL; Amersham Biosciences).

**Kinase Activity**—Kinase activity was measured by using a p44/p42 MAPK assay kit (Cell Signaling), following the manufacturer's protocol. In brief, 250  $\mu$ g of lysate from PDGF-BB or vehicle treated SMC was incubated with 15  $\mu$ g of provided immobilized phospho-p44/p42 MAPK antibody, and complexes were rotated overnight at 4 °C. Immune complexes were collected by centrifugation and washed three times with provided 1 $\times$  lysis buffer and resuspended in provided 1 $\times$  kinase buffer containing 200  $\mu$ M ATP and 2  $\mu$ g of ELK-1 fusion protein. The reaction was incubated at 30 °C for 30 min and terminated by the addition of 3 $\times$  SDS sample buffer. Sample proteins were electrophoresed on an 11% SDS-PAGE gel, transferred to a nitrocellulose membrane, incubated with phospho-ELK-1 antibody, and processed as described above.

**GST Pull Down Assay**—GST-Pak (amino acid 1–290 of Pak1) was purified from bacterial lysates using glutathione-agarose beads (Amersham Biosciences) as described previously (16). GFP- or GFRNK-infected SMC were serum-starved and treated with PDGF-BB or Ang II as described above. Cells were lysed in Buffer A (50 mM Tris, pH 7.6, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 0.5 mM MgCl<sub>2</sub>, plus 100  $\mu$ M leupeptin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, and 0.05 trypsin inhibitory units/ml aprotinin) and cleared by centrifugation for 10 min at 14000 rpm at 4 °C (17). 500  $\mu$ g of lysate was combined with 30  $\mu$ g of GST-PAK, and samples were rotated at 4 °C for 30 min. The complexes were pelleted by centrifugation and washed two times in Buffer B (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5 mM MgCl<sub>2</sub>, plus 100  $\mu$ M leupeptin, 1 mM AEBSF, and 0.05 trypsin inhibitory units/ml). Samples were boiled in SDS-PAGE buffer, electrophoresed (15% SDS-PAGE gel), and transferred to polyvinylidene difluoride (Bio-Rad). Western blotting was performed using an anti-Rac1 primary antibody as described above.





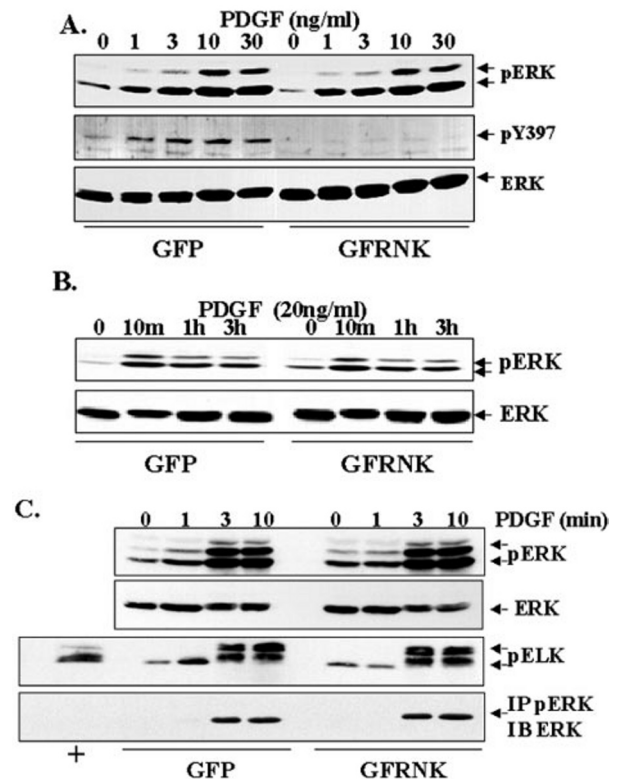
**FIG. 1. FRNK inhibits FAK, p130CAS, and paxillin phosphorylation in SMC.** Rat aortic SMC were infected with GFP or GFRNK adenovirus for 12 h. Extracts (100  $\mu$ g) were analyzed by SDS-PAGE and immunoblotting using a generic anti-phosphotyrosine antibody (4G10) (panel A), an antibody to total FAK, or phosphorylation-specific antibodies to pY397, pY576, pY577, and pY861 of FAK (panel B). C, paxillin (PAX) or p130Cas (CAS) were immunoprecipitated (IP) from the GFP- or GFP-FRNK-infected cell extracts as described under "Experimental Procedures" followed by immunoblotting with anti-phosphotyrosine antibody (4G10) and the appropriate antibody for loading controls.

## RESULTS

We reported previously that overexpression of GFP-FRNK in cultured aortic SMCs by adenoviral gene transfer resulted in a modest increase in FRNK protein effectively inducing an exogenous FRNK to endogenous FAK ratio of around 3:1. This FRNK:FAK ratio compares favorably with endogenous ratios observed in neonatal vessels and is sufficient to inhibit both FAK activity and PDGF-BB-stimulated proliferation and migration. To begin to dissect the signaling pathways altered by FRNK overexpression in SMC, we first examined the effect of FRNK overexpression on total cellular phosphotyrosine levels. In adherent cultures of SMC, three major tyrosine phosphorylated bands were attenuated in GFRNK in comparison with GFP-infected or uninfected control cells (Fig. 1A). The 125-kDa protein is probably FAK because FAK co-migrates with this band, and FRNK overexpression in SMC inhibits autophosphorylation of Tyr-397 as reported previously (12). The use of additional phosphospecific antibodies demonstrated that FRNK overexpression in SMC also inhibited phosphorylation of the major Src-dependent phosphorylation sites in FAK namely pY556, pY557, and pY861 (Fig. 1B). The tyrosine-phosphorylated proteins migrating at  $\sim$ 130 and 70 kDa likely represent the FAK binding partners p130Cas and paxillin (Fig. 1C).

Because proliferation and migration of SMC in response to PDGF-BB are at least partially dependent on ERK activation (18–20), and because ERK activation has been reported to be dependent on FAK/p130Cas interactions (21), we tested whether overexpression of GFP-FRNK could attenuate mitogen-stimulated ERK activity. Surprisingly, dose-dependent stimulation of ERK by PDGF-BB was not altered by overexpression of GFP-FRNK (Fig. 2A, top panel) even though PDGF-BB-stimulated FAK activation (in the same lysates) was clearly attenuated (Fig. 2A, middle panel).

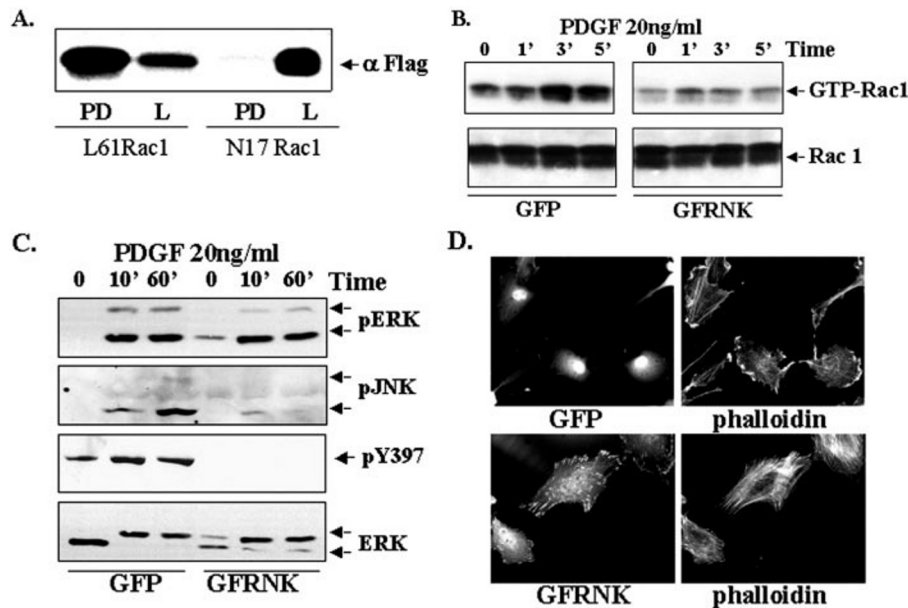
Because the time course of ERK1/2 activation may also regulate cell cycle progression, (22, 23) we examined whether FRNK might alter the kinetics of PDGF-BB-stimulated ERK activity. As shown in Fig. 2, B and C, FRNK expression did not alter the kinetics of PDGF-BB-induced ERK activation at time points ranging from 1 min to 3 h. In addition, we observed no significant difference in the ability of ERK to phosphorylate ELK-1 as assessed by an immune-complex kinase assay that measures direct phosphorylation of a GST-ELK fusion protein (Fig. 2C). These data likely indicate that in SMC, FRNK inhib-



**FIG. 2. FRNK expression does not alter dose- or time-dependent activation of ERK by PDGF-BB in SMC.** Rat aortic SMC were infected with GFP or GFP-FRNK adenovirus (multiplicity of infection, 10) for 12 h. Cells were transferred to serum-free medium and incubated for 4 h. Cells were treated with PDGF-BB (0–30 ng/ml) for 10 min (panel A) or with PDGF-BB (20 ng/ml) for various times as indicated (panel B). Lysates were electrophoresed, and Western analysis was performed using anti-ERK, or anti-phosphospecific ERK and FAK (pY397) Ab. Panel C, ERK was immunoprecipitated (IP) from cell lysates with an anti-ERK1/2 antibody, and immune complexes were washed and incubated with 2  $\mu$ g of purified GST-ELK immobilized on glutathione beads in a kinase buffer containing 10 mM MgCl<sub>2</sub> and 200  $\mu$ M ATP for 30 min at 30  $^{\circ}$ C. Immune complexes were then electrophoresed, and Western analysis was performed using a phosphorylation-specific ELK-1 antibody. The plus sign (+) indicates a positive control whereby 20 ng of active ERK was added to the reaction. Control blots indicated that FRNK expression significantly attenuated FAK phosphorylation as assessed by Western blotting with an anti pY397 antibody in each experiment. Data are representative of at least three separate experiments.

its PDGF-BB-stimulated migration and proliferation in an ERK-independent fashion.

Other major signaling pathways known to regulate both proliferation and migration include activation of the small GTPases of the Rho family. Interestingly, Rac1 has been shown previously (24, 25) to be activated both by PDGF receptors and following engagement of certain integrins. We used a well defined assay to examine Rac1 activation in SMC using a GST-PAK1 fusion protein to precipitate active (GTP-bound) but not inactive (GDP-bound) Rac1. As shown in Fig. 3A, this GST fusion protein effectively precipitated activated L61Rac but not inactive N17Rac when these constructs were overexpressed in COS-7 cells. Using this assay, we have shown that PDGF-BB induced a rapid transient activation of Rac1 in SMC. PDGF stimulated an approximate 2–3-fold increase in Rac1 activity, which peaked between 1 and 3 min following stimulation (Fig. 3B, left panel). As shown in Fig. 3B, right panel, overexpression of GFP-FRNK slightly inhibited basal activity and markedly attenuated PDGF-stimulated Rac1 activity in SMC. In addition, activation of JNK (a kinase known to be downstream of Rac1 activation) was also attenuated in the



**FIG. 3. FRNK expression inhibits PDGF-BB-stimulated Rac1 activation and membrane ruffling in SMC.** *A*, COS cells were transfected with FLAG-tagged active (*L61*) Rac1 or dominant-negative (*N17*) Rac1 for 48 h. Lysates were prepared and incubated with 30  $\mu$ g of purified GST-PAK1 immobilized on glutathione beads for 30 min. Beads were washed, boiled in sample buffer, electrophoresed, and Western blotted using an anti-FLAG Ab. *PD* indicates the Rac1 precipitated with GST-PAK, and *L* indicates a 10% loading control. *B*, rat aortic SMC were infected with GFP or GFP-FRNK adenovirus (multiplicity of infection, 10) for 12 h. Cells were transferred to serum-free medium and incubated for 4 h. Cells were treated with 20 ng/ml PDGF-BB for the indicated times. Lysates were incubated with 30  $\mu$ g of purified GST-PAK for 30 min, complexes were washed, and Western blotting was performed using an anti-Rac1 Ab. The *bottom panels* represent a 10% loading control of total Rac1 in the lysates. *C*, rat aortic SMC were infected and treated with PDGF-BB (20 ng/ml) as described above for times indicated. Lysates were electrophoresed, and Western analysis was performed using phosphorylation-specific anti-JNK, anti-ERK, or anti-FAK (*pY397*) antibodies. The blot was stripped and re-probed using an anti-ERK Ab to serve as a loading control. *D*, rat aortic SMC were infected with GFP or GFP-FRNK adenovirus and serum-starved as described above. Cells were treated with 20 ng/ml PDGF-BB for 45 min. Cells were fixed and stained with Texas Red-conjugated phalloidin to visualize filamentous actin as described under "Experimental Procedures." The ruffled phenotype was observed in 87% of GFP-infected cells compared with 22% of FRNK-infected cells (187/215 and 46/205 cells, respectively). Data shown are representative of at least three separate experiments.

GFP-FRNK expressing cells (Fig. 3*C*). Because Rac1 activation stimulates the formation of distinct actin-rich lamellipodia, an important event during cell motility (24, 26, 27), we examined whether FRNK expression in SMC might alter this process in PDGF-BB-stimulated SMC. Indeed, in GFP-infected cells, PDGF-BB stimulated a reorganization of actin filaments and a resultant ruffled morphology (note the dissolution of stress fibers and formation of actin bundles around the periphery of the PDGF-BB-stimulated cells; see Fig. 3*D*, *top panels*). In contrast, PDGF treatment of GFP-FRNK-infected cells did not result in noticeable changes in actin rearrangements (Fig. 3*D*, *bottom panels*). Taken together, these data indicate that FRNK expression attenuates the activation of Rac1 and Rac1-dependent signals in SMC.

To determine whether inhibition of Rac1 was the sole determinant for the proliferative defects induced by FRNK, we examined the ability of activated Rac1 to rescue PDGF-BB-stimulated proliferation in FRNK overexpressing cells. To this end, SMC were transfected with either DsV or DsRacL61 prior to infection with GFP and GFP-FRNK adenoviruses. Following serum starvation, cells were treated with PDGF and analyzed for their capacity to incorporate BrdUrd. Consistent with our previous report, FRNK overexpression attenuated PDGF-stimulated BrdUrd incorporation by ~40% to a level comparable with cells in serum-free media ( $p < 0.05$  compared with GFP-infected cells; see Fig. 4). Importantly, although Rac1 alone had little effect on BrdUrd incorporation in GFP expressing cells, overexpression of constitutively active L61Rac1 efficiently rescued the effects of FRNK on cell proliferation ( $p < 0.01$  compared with FRNK + DsV). Control experiments indicated that similar amounts of FRNK were expressed under both condi-

tions (Fig. 4, *upper panel*). These data indicate that Rac1 acts downstream of FAK/FRNK to control SMC proliferation.

To determine whether inhibition of Rac1 activity might be a general mechanism by which FRNK attenuates SMC growth, we examined the capacity of FRNK to inhibit Ang II-mediated signal transduction. We found that overexpression of FRNK also inhibits Ang II-stimulated cell growth reducing the proliferative index by  $77 \pm 12\%$  after a 24-h treatment as assessed colorimetrically by monitoring cleavage of the tetrazolium salt, WST-1. These data corroborate studies reported elsewhere (28) and suggest that FRNK can inhibit proliferative signals induced by G protein-coupled receptors, as well as receptor tyrosine kinases. Because Ang II has also been shown to stimulate Rac1 activity in SMC (29) and other cell types, we examined whether this effect might also be altered by FRNK. Indeed, as shown in Fig. 5*A*, Ang II stimulated a rapid transient increase in Rac1 activity in SMC, and this effect was attenuated by ectopic expression of GFP-FRNK. As shown in Fig. 5, *B* and *C*, neither the kinetics of Ang II-stimulated ERK activation nor the concentrations of Ang II required for maximal ERK activation were significantly altered by overexpression of GFP-FRNK in SMC. Control experiments indicated that Ang II does stimulate FAK activity and that this activity is blocked by overexpression of FRNK. Thus, FRNK appears to attenuate both Ang II- and PDGF-BB-dependent mitogenic signaling without effects on ERK activity.

As noted above signaling through many SMC mitogens is dependent on ECM, but the precise mechanisms involved in this regulation have not been clearly defined. In certain cell types, fibronectin-binding integrins have been shown to contribute to the activation of Rac1 (30). Thus, we examined

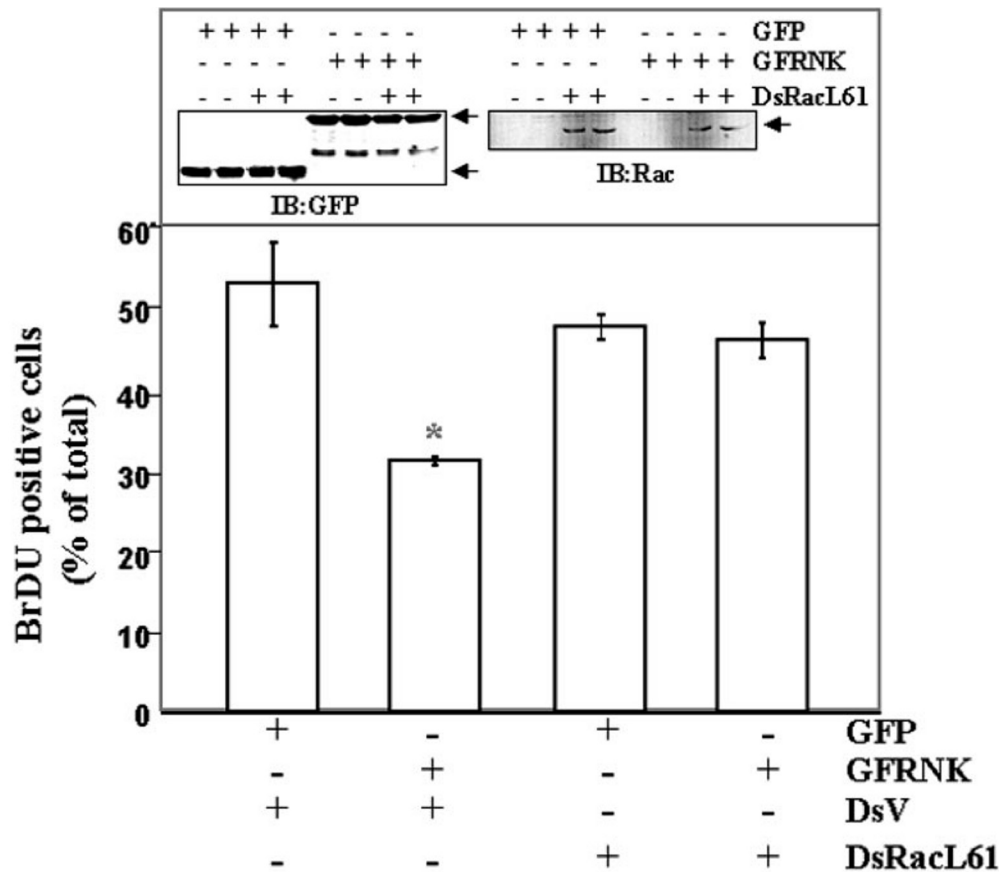


FIG. 4. **L61Rac1 expression overcomes inhibitory effect of FRNK on proliferation.** SMC were transfected with DsV or a DsRed fusion protein containing the constitutively active form of Rac1 (*DsRacL61*). 48 h after transfection, cells were infected with GFP or GFP-FRNK virus as described above. Cells were serum-starved for 4 h and then dosed overnight with either 30 ng/ml PDGF-BB or vehicle control. Cells were labeled with BrdUrd, fixed, and stained for BrdUrd using a cascade blue-conjugated secondary antibody as described under "Experimental Procedures." The BrdUrd labeling index for cells positive for GFP and DsRed was determined, and data are presented as the percentage of cells labeled positive in the PDGF-BB-stimulated *versus* unstimulated wells. The graph represents means  $\pm$  S.E. of three independent experiments in which a total of 180–350 cells were counted for each condition. The percentage of BrdUrd-positive cells in the unstimulated GFP-FRNK control group was  $18.2 \pm 1.9\%$ . Levels of GFP-FRNK were comparable in DsV and DsRacL61 expressing cells (see upper panel).

whether plating SMC on fibronectin would stimulate the activation of Rac1 in SMC and if so, whether expression of FRNK might alter integrin-dependent Rac1 activation. As shown in Fig. 6, plating SMC on fibronectin stimulated a transient activation of Rac1 that peaked at 1 h, and overexpression of FRNK attenuated this response at each time point (Fig. 6). In contrast, overexpression of FRNK had little effect on fibronectin-stimulated ERK activity. These data indicate that FRNK attenuates FAK-dependent activation of Rac1 in response to PDGF-BB, Ang II, and integrins. Based on these observations, we hypothesize that Rac1 activation may be a key convergence point in growth factor and integrin-regulated cell proliferation and migration in SMC and that FRNK may act to regulate signaling through diverse pathways at this level (see Fig. 7).

#### DISCUSSION

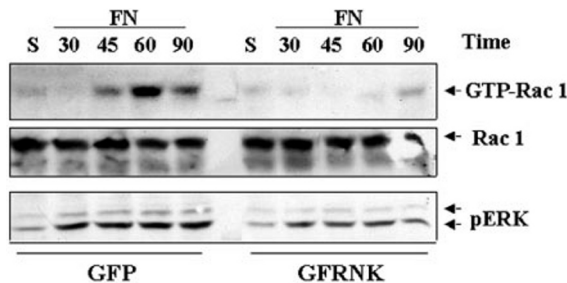
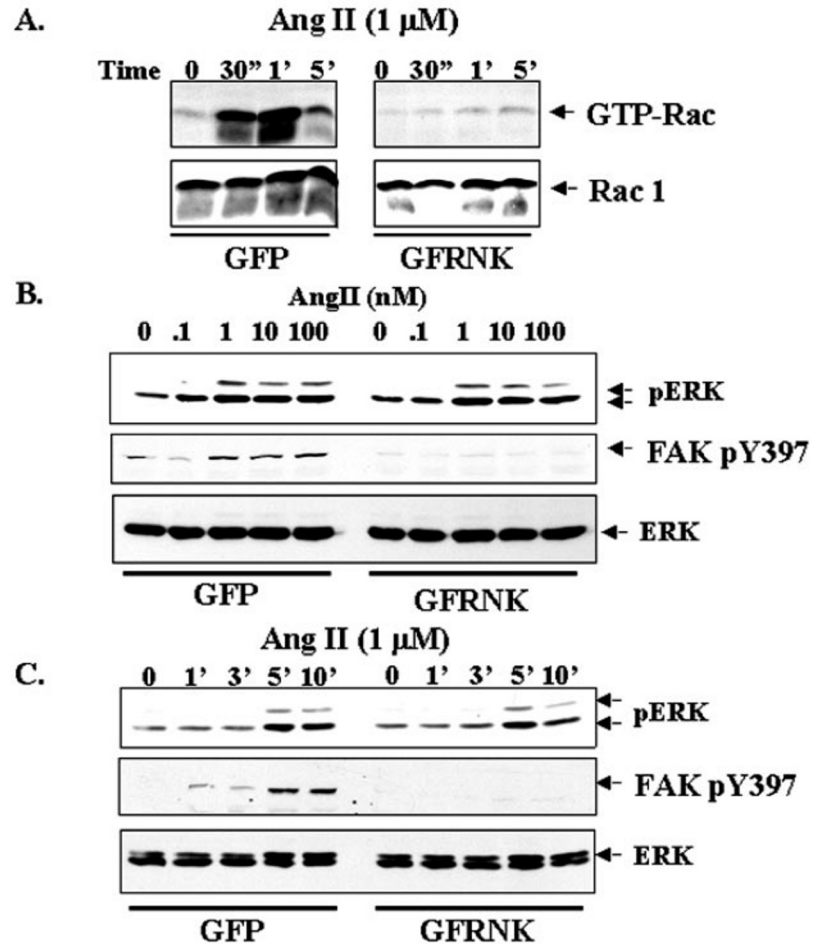
Numerous studies have implicated a role for the non-receptor tyrosine kinase, FAK, in the regulation of migration and proliferation. FAK is strongly activated by integrin-, growth factor receptor-, and G protein-coupled receptor engagement and thus may serve to integrate downstream signals from a variety of agonists; however, the precise mechanisms by which FAK regulates signaling from these diverse pathways have not been clearly defined (11). In this report, we studied the convergence of growth factor and integrin signaling through FAK in the regulation of vascular SMC proliferation. As we reported previously (12, 31, 32), FAK activity appears to be regulated in

a unique fashion in SMC whereby autonomous expression of the C-terminal domain of FAK (termed FRNK) can act as an endogenous inhibitor of FAK signaling. In recent studies, we have shown the following: 1) that FRNK is expressed selectively in SMC with high levels observed in the vasculature; 2) that FRNK expression is regulated during development and following vascular injury; and 3) that ectopic expression of FRNK in cultured SMC blocked PDGF-BB-stimulated migration and proliferation (12). These data indicate that FRNK expression may impart tight regulation on FAK-dependent ECM-mediated signaling events in SMC. Here we report that expression of FRNK in SMC blocks mitogenic signaling from diverse factors by attenuating FAK-dependent co-activation of the Rac/JNK but not the Ras/ERK pathway.

Ras/ERK and Rac/JNK are two of the main pathways known to be key regulators of cell proliferation and migration. Cell adhesion to extracellular matrix regulates Ras/ERK signaling in many cell types, and studies have clearly shown that maximal growth factor signaling to ERK is anchorage-dependent (33). Several pieces of evidence highlight a possible role for FAK activation in ERK-dependent growth regulation. First, several mitogens (including PDGF-BB, Ang II, phenylephrine, IGF-1, and ET-1) stimulate FAK tyrosine phosphorylation in an adhesion-dependent manner (5–8). Second, at least two pathways have been defined by which FAK can activate ERK activity. One pathway involves adhesion-dependent tyrosine phosphorylation of FAK at residue 925, which directs Src ho-



**FIG. 5. FRNK expression attenuates Ang II-stimulated Rac1 but not ERK activity in SMC.** Rat aortic SMC were infected with GFP or GFP-FRNK adenovirus (multiplicity of infection, 10) for 12 h. Cells were transferred to serum-free medium and incubated for 4 h. Cells were treated with Ang II for various times (panels A and B) or with various concentrations (panel C). A, lysates were incubated with 30  $\mu$ g of purified GST-PAK as described above. Western blotting was performed using an anti-Rac1 Ab. The bottom panels represent a 10% loading control of total Rac1 in the lysate. B and C, lysates were electrophoresed, and Western analysis was performed using an anti-ERK or anti-phosphospecific ERK Ab. Control blots indicated that FRNK expression significantly attenuated FAK pY397 in each experiment. Data in panel A are representative of three separate experiments, and data in panels B and C are representative of at least four separate experiments.



**FIG. 6. FRNK expression blocks FN-stimulated Rac1 activity in SMC.** SMC were infected with GFP or GFP-FRNK as described above. Cells were trypsinized, neutralized in soybean trypsin inhibitor, pooled, and either held in suspension (S) or plated on fibronectin (FN) for the times indicated. Cells were lysed and incubated with 30  $\mu$ g of purified GST-PAK as described above. Western blotting was performed using an anti-Rac1 Ab. The middle panel represent a 10% loading control of total Rac1 in the lysate. Levels of active ERK in lysates were assessed using an anti-phosphospecific ERK Ab (bottom panel). Data are representative of three separate experiments.

mology 2-mediated binding to the adapter protein Grb2, and recruitment of the GTP exchange factor Sos followed by subsequent activation of the Ras/Raf/MEK/ERK pathway (34, 35), and a separate pathway involves the adhesion-dependent association of the adapter protein CAS with FAK, which can facilitate ERK signaling through the adapter proteins Nck and Sos and subsequent activation of the Ras/Raf/MEK/ERK pathway (21). Finally, overexpression of FAK can rescue growth factor-induced ERK activation in non-adherent cells whereas inhibition of FAK function can attenuate activation of ERK in adherent cells (9, 36). Indeed, we reported previously (37) that

adenoviral infection of GFP-FRNK inhibits phenylephrine-stimulated ERK activation in isolated cardiomyocytes.

In contrast, we have clearly shown in this report that FRNK expression attenuates FAK signaling in primary cultured SMC (in which FRNK is expressed endogenously) without effect on the time- or dose-dependent activation of ERK by both strong (PDGF-BB) or weak agonists (Ang II). Instead, the effects of FRNK on mitogen- and ECM-induced proliferation were mediated by inhibition of Rac1/JNK, a conclusion strongly supported by our results demonstrating that L61 Rac1 was sufficient to rescue the FRNK inhibitable component of PDGF-BB-stimulated cell proliferation. Our results do differ somewhat from a previous report in which stable transfection of FRNK into smooth muscle-like cells reduced PDGF-stimulated ERK activity at low but not high concentrations of PDGF (38). The apparent discrepancies between these results may be because of their use of smooth muscle cell lines immortalized by large T antigen as opposed to primary SMC cultures. Thus, we propose that the mechanisms by which FAK regulates adhesion-dependent growth factor signaling may be cell type-specific. Indeed, previous work (39) has shown that in keratinocytes, integrin-dependent ERK activation appears to proceed through SHC- but not FAK-dependent signals. Perhaps similar FAK-independent ERK signaling pathways are utilized in SMC. Although most of our data indicate that the effects of FRNK are mediated by inhibition of FAK, we cannot rule out the possibility that FRNK might regulate signaling in a FAK-independent fashion in SMC.

Our results do not completely exclude the possibility that FAK/FRNK may play a role in regulating ERK signaling in SMC *in vivo*. It is still possible that weak ERK activation

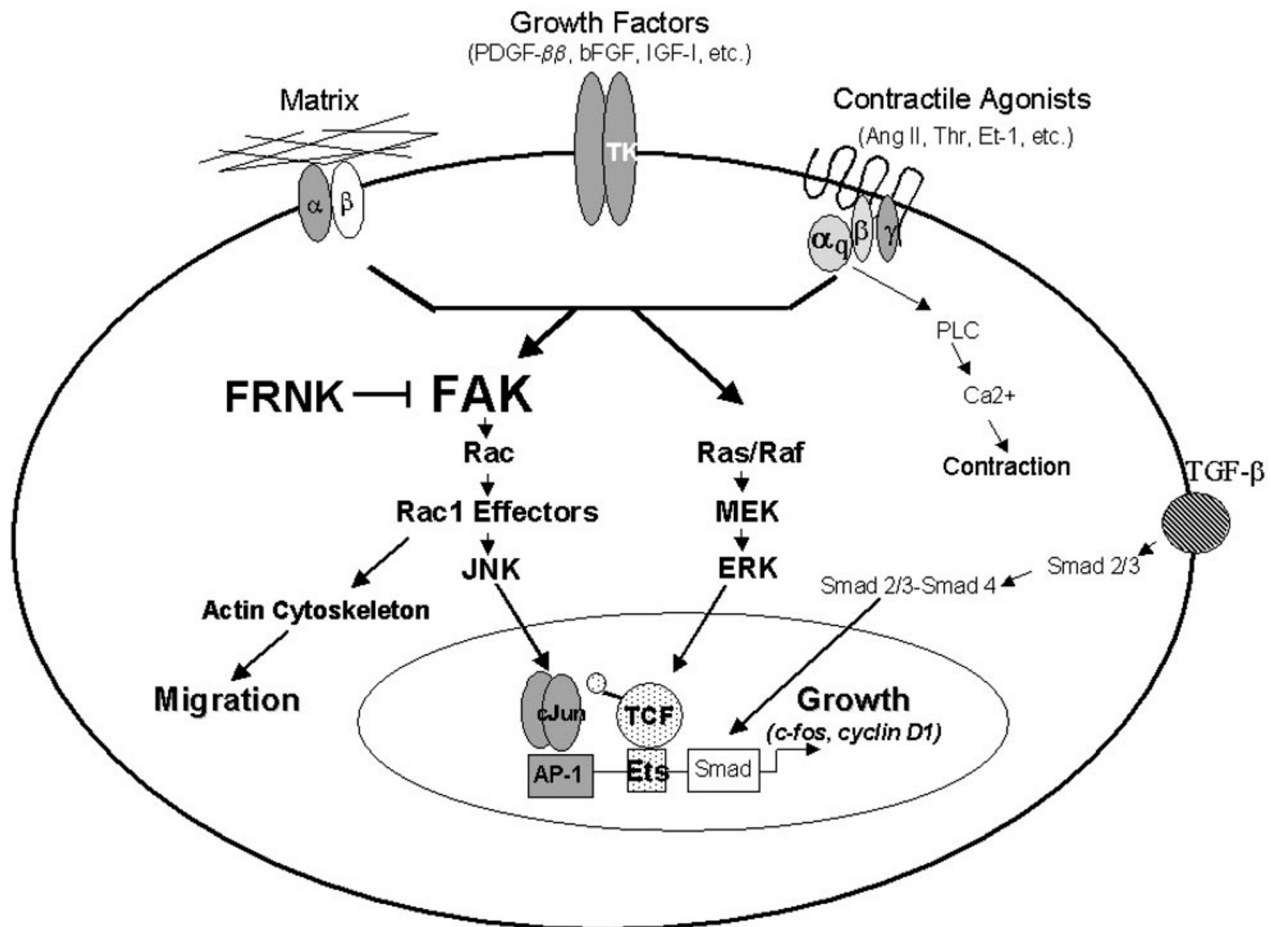


FIG. 7. Schematic summarizing mitogenic signaling pathways that regulate SMC growth and migration. A variety of agonists that regulate SMC growth and migration are known to regulate Rac/JNK and Ras/ERK signaling. We hypothesize that FAK activation is required to integrate signals from integrins, receptor tyrosine kinases, and G protein-coupled receptors leading to the downstream activation of Rac1. In SMC, FRNK may control proliferation and migration by buffering this FAK-dependent response.

induced by limiting amounts of mitogens in the vessel wall may be more susceptible to the effects of FRNK. It is also possible that examination of total ERK activity may not be representative of the relevant functional activity of this enzyme in the cell. For instance, active ERK localizes to focal adhesions, as well as the nucleus, suggesting that targeting of this kinase may direct specificity toward downstream targets (40, 41). Thus, FRNK may attenuate localization of active ERK, which could be masked in experiments designed to measure total activity in cell lysates. Interestingly, adhesion to the ECM is required for efficient accumulation of active ERK in the nucleus, and recent studies (40, 42) point to a role for Rac1 in mediating this process. Therefore, although FRNK might not affect ERK activation *per se*, it could affect downstream ERK signaling secondary to its effect on Rac1 activation. Studies to examine this possibility are currently underway.

Our data suggest that in SMC, Rac1 activation may be a key convergence point in growth factor- and integrin-regulated cell proliferation, because FRNK expression attenuated integrin-, Ang II-, and PDGF-BB-stimulated Rac1 activation and downstream signaling. Activation of the small GTPase Rac1 elicits membrane ruffling and lamellipodia formation upon growth factor stimulation, and this modification of cell morphology is required for cell spreading and migration (24, 26, 27). In addition, activation of Rac1 is required for cyclin D1 expression and concomitant cell cycle progression (43, 44). Rac1, like other low molecular GTPases, is a molecular switch that cycles between the active GTP- and inactive GDP-bound states and is regu-

lated by numerous proteins that facilitate this process. The exchange of GDP for GTP is accelerated by guanine nucleotide exchange factors, whereas GTPase-activating proteins increase the intrinsic rate of GTP hydrolysis. In the GTP-bound form, Rac1 interacts with and activates a number of effector molecules that have been implicated in regulating proliferation and migration including serine/threonine protein kinases, lipid kinases, and actin binding/scaffolding proteins (45, 46). We have shown that FRNK expression attenuates PDGF-BB-mediated activation of JNK, a stress-activated protein kinase downstream of Rac1 signaling known to control cell proliferation. In addition, we showed that the proliferative defect in FRNK expressing cells was completely rescued by active Rac1L61. Taken together, these data provide strong evidence that Rac1 is co-regulated by FAK-dependent integrin and growth factor signaling.

Recent studies corroborate the suggestion that activation of the Rac1 GTPase may be the critical convergence point for mitogen- and integrin-dependent growth and migration in certain cell types. In endothelial cells, mitogens stimulate robust ERK activity when cells are attached to either fibronectin or laminin but only stimulate proliferation when the cells are plated on fibronectin. This effect was correlated to the ability of fibronectin (but not laminin) to support mitogen-stimulated Rac1 activity, indicating a role for Rac1 in adhesion-dependent growth responses (30). Two other reports show that  $\beta$  integrins are both necessary and sufficient for adhesion dependent Rac1 activation. Hirsch *et al.* (47) showed that fibroblasts derived

from mice that express a mutated  $\beta_1$  integrin display defective activation of FAK, Rac1, and JNK but not ERK and are impaired in G<sub>1</sub>-S cell cycle progression. In addition, Berrier *et al.* (48) showed that clustering  $\beta_1$  and  $\beta_3$  integrin tails on the surface of non-adherent cells activates Rac1 activity.

FAK activates a number of signaling molecules that might contribute to integrin-dependent Rac1 (and downstream JNK) activation. Evidence suggests that FAK-dependent signaling through CAS, paxillin, or PI3K can lead to activation of Rac1 by several different mechanisms. Integrin-mediated activation of Rac1 could proceed through a FAK/(CAS or paxillin)/Crk/DOCK 180 pathway. Indeed, dominant interfering mutants of the Src homology 2/Src homology 3 adapter protein Crk, the adapter/exchange factor DOCK 180, or Rac1 each attenuate Cas- or paxillin-stimulated cell migration (49–52). Recently, a more direct paradigm has been suggested whereby FAK-dependent activation of paxillin could lead to focal adhesion recruitment and activation of the putative Cdc42/Rac guanine nucleotide exchange proteins PIX/COOL (3, 30, 53). Alternatively, activation of Rac1 could proceed through a FAK/PI3K/Sos pathway. PI3K associates with Tyr-397 of FAK upon integrin ligation and one of its catalytic products, phosphatidylinositol triphosphate, binds to and activates the PH domain-containing guanine nucleotide exchange factor, Sos, which can activate both Rac1 and Ras (53, 54). In endothelial cells fibronectin-stimulated Rac1 activity is dependent on FAK/PI3K/Sos signaling (30); however, recent data using Src-transformed fibroblasts suggest that a FAK/CAS/DOCK 180 pathway might be utilized (55). Whether the pathways leading from FAK to Rac/JNK are redundant or differentially regulated in a cell type-specific fashion are important questions that remain to be addressed.

ECM is a critical component of the medial layers of the vessel wall that serves to both maintain vascular integrity and to regulate vascular development and remodeling. Results from genetic deletion of various ECM components, integrin receptors, and FAK each result in extraembryonic and embryonic vessel defects leading to lethality at approximately day 8.5–10 of mouse development supporting a role for adhesion signaling in the regulation of growth and migration of vascular SMC (56–59). FAK is co-activated by integrins and autocrine factors, and its activation is necessary for proliferation and migration in a number of cell types (11). We have shown that FAK activity may be regulated in a unique fashion in SMC, because FRNK, a dominant inhibitory form of FAK, is selectively expressed in these cells. FRNK overexpression inhibits both Ang II- and PDGF-BB-stimulated migration and proliferation, effects that are likely due to the ability of FRNK to block Rac1 activation. Interestingly, several SMC mitogens have been linked to Rac1 activation in either SMC (*i.e.* Ang II) or other cell types (*i.e.* PDGF, ET-1, IGF-1, fibroblast growth factor), and proliferative signals induced by these agonists are attenuated by overexpression of a dominant interfering mutant for Rac1 (29, 60–64). Thus, it is tempting to speculate that enhanced signaling through FAK to Rac1 is permissive for SMC growth during development and for the phenotypic reversion of SMC to a more proliferative state following vascular injury and that expression of FRNK might hold these processes in check.

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